b.) Remarks

Claim 51 is objected to as containing a typographical error. Such formal matter has been attended to above, in conformity with the Examiner's kind suggestion.

Claims 3-4 and 50 are rejected under 35 U.S.C. 112, first paragraph, because the Examiner contends the specification does not reasonably enable one of ordinary skill to practice the scope of the invention. The Examiner states (page 7, line 18 to page 8, line 1) that without guidance, random screening is excessive.

At the outset, Applicants respectfully wish to point out this is not what *Ex parte Forman* says; rather the specification is enabling if it presents a reasonable amount of guidance or if random experimentation is routine in the art (230 USPQ at 547). In this regard, the Examiner has made no *prima facie* showing that the required level of experimentation, even if random, is not routine. Indeed, it plainly is where, as here, such can simply be automated. Nonetheless, solely in order to reduce the issues and expedite prosecution, Applicants have above cancelled these claims without prejudice or disclaimer. Accordingly, the rejection is mooted.

Claims 2-9, 11-13 and 48-52 are rejected under 35 U.S.C. 102(a) as being anticipated by Isshiki et al. (J. Biol. Sci. Vol, 274(18):12499-12507, Apr. 1999) or Zhou D. et al. (Eur. J. Biochem. Vol. 263(2):571-576). Claim 10 is rejected under 35 U.S.C. 103(a) as being unpatentable over Isshiki et al. or Zhou D. et al.

Initially, it is noted that Isshiki is dated "April 1999", which date follows the February 25, 1999 filing date of Applicants' Japanese priority application JP 99-47571. Additionally, Applicants' investigation has determined that Zhou was not published, at the

earliest, until July 11, 1999.[1/]

The rejections over both Isshiki and Zhou can be overcome by filing a sworn translation of Applicants' Japanese priority application. Accordingly, such translation is enclosed.

In view of the above amendments and remarks, and the accompanying sworn translation, Applicants submit that all of the Examiner's concerns are now overcome and the claims are now in allowable condition. Accordingly, reconsideration and allowance of this application is earnestly solicited.

Claims 2, 5-25, 29-31, 33, 38, 48, 49 and 51-54 remain presented for continued prosecution. Rejoinder of claims 14-25, 29-31, 33, 38, 53 and 54, each of which relate to a process of using (claims 18-25, 53 and 54), producing (claims 14-17) or determining (claims 29-31, 33 and 38) the elected subject matter, is respectfully requested

---

1/ Applicants have learned Zhou was publicly available, according to the publisher, on July 12, 1999 (Tab A). It was first received in the Senckenberg library on July 26, 1999, and was not published online until December 25, 2001 (Tab B). The reference itself is dated July 11, 1999 (Tab C).

Applicants' undersigned attorney may be reached in our New York office by telephone at (212) 218-2100. All correspondence should continue to be directed to our below listed address.

Respectfully submitted,

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Wolfgang Völger

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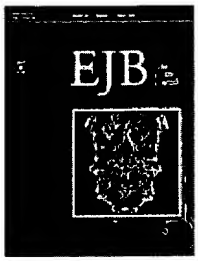

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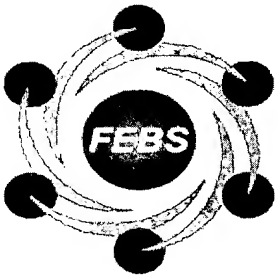

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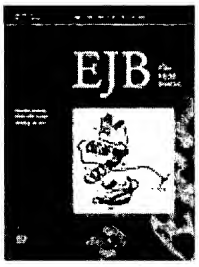

Molecular cloning of a human UDP-galactose:GlcNAc β1,3GalNAc β1,3 galactosyltransferase gene encoding an O-linked core3-elongation enzyme

Dapeng Zhou, Eric G. Berger and Thierry Hennet

Using the full-length amino-acid sequences of the human β1,3 galactosyltransferase (β3GalT)-I, -II and III enzymes as query, we have identified an additional member of the β3GalT gene family within a sequenced region of the human chromosome 21 as found in GenBank. The novel human β3GalT-V gene included an open reading frame of 933 bp encoding a protein of 310 amino acids with a short N-terminal cytoplasmic tail, a single predicted transmembrane domain and a large lumenal catalytic domain. The human β3GalT-V protein showed 34%, 27%, 31% and 23% sequence identity with the human β3GalT-I, -II, -III and -IV enzymes, respectively. The expression of β3GalT-V as a recombinant protein in Sf9 insect cells confirmed the galactosyltransferase activity catalyzed by this enzyme. Similarly to β3GalT-I, -II and -III, the β3GalT-V enzyme used β-linked GlcNAc as an acceptor, but unlike the former enzymes β3GalT-V exhibited a marked preference for the O-linked *core3* GlcNAcβ1,3GalNAc substrate. The β3GalT-V gene was mainly expressed in human small intestine and to a lesser extent in pancreas and testis. Although β3GalT-V transcripts were not detected in normal colon tissue, based on Northern analysis, β3GalT-V mRNA was found in the adenocarcinoma cell line Colo 205.

The availability of large DNA sequence databases has enabled the retrieval of structurally related genes based on similarity searches. This approach has been fruitful in glycobiology, as many glycosyltransferase activities previously believed to be encoded by single genes have turned out to represent families including several enzymes. Characterization of these families has revealed the complex pattern of tissue expression of the corresponding genes and the specificity of each protein towards distinct acceptor substrates, as exemplified by fucosyltransferase [1] and polypeptide N-acetylgalactosaminyltransferase enzymes [2].

In vertebrates, galactose is transferred either as α- or β-anomer through 1,3 or 1,4 linkage (reviewed in [3]). Multiple β1,4 galactosyltransferase (β4GalT) [4-8] and α1,3 galactosyltransferase (α3GalT) genes [9, 10] have been identified. Similarly, four β1,3 galactosyltransferase (β3GalT) genes have been characterized recently [11-14]. β3GalT enzymes catalyze the formation of various structures of biological importance. For example, type 1 chain resulting from the transfer of galactose in a β1,3 linkage to GlcNAc constitutes the base of the Galβ1,3(Fucα1,4)GlcNAc Lewis Le^a and Fucα1,2Galβ1,3(Fucα1,4)GlcNAc Le^b antigens (reviewed in [15]). Also, galactose β1,3 linked to GalNAc, a structure known as the Thomsen-Friedenreich antigen [16] enables

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the formation of *core2* structures of O-glycans [17]. Among the known β3GalT enzymes, three catalyze the transfer of Gal to GlcNAc-based acceptors. Here, we report the characterization of an additional β3GalT enzyme by searching for sequences similar to β3GalT-I to -III proteins. The novel β3GalT enzyme, named β3GalT-V, displayed a marked preference for the O-linked *core3* GlcNAcβ1,3GalNAc acceptor structure. Accordingly, the β3GalT-V enzyme likely represents the β3GalT previously isolated from pig trachea [18].

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Materials and methods Go to: Choose GO

Cloning of the β3GalT-V gene and baculovirus expression

The protein-coding region of the β3GalT-V gene was amplified by PCR from human genomic DNA using the upstream primer 5'-GATTTGGATCCTTTCAGATGGCTTTC-3' and the downstream primer 5'-TGTGCCTCGAGGCTCCCCTCAGACAG-3' that included *Bam*HI and *Xho*I restriction sites, respectively. The PCR conditions were three cycles at 94 °C for 45 s, 50 °C for 30 s and 72 °C for 60 s, followed by 25 cycles at 94 °C for 45 s, 58 °C for 30 s, 72 °C for 60 s. The resulting 956-bp fragment was subcloned into the *Bam*HI-*Xho*I sites of the pFastBac1 donor vector (BAC-to-BAC system, Life Technologies), which was subsequently processed as recommended by the manufacturer to generate pure recombinant baculoviruses. Sf9 insect cells were infected at a multiplicity of 10 and incubated at 27 °C for 72 h.

Galactosyltransferase assay

The carbohydrate acceptors and the donor UDP-Gal were purchased from Sigma. Sf9 cells were lyzed in 2% Triton X-100 for 15 min on ice and enucleated by centrifugation at 500 ***g*** . Galactosyltransferase activity was assessed using 10 μL of Sf9 cell lysate in 50 μL assays of 50 m M cacodylate buffer, pH 7.0, 10 m M $MnCl_2$, 0.2 m M UDP-Gal, 5×10^4 c.p.m. of UDP-[^{14}C]Gal (82 pmol) (Amersham), and various acceptors (Table 1). Samples were incubated at 37 °C for 60 min and reactions were stopped by adding 500 μL of cold H_2O. For kinetic analysis, the assays were performed using 0.5 m M UDP-Gal and 10^5 c.p.m. of UDP-[^{14}C]Gal (164 pmol); reactions were stopped after 30 min. Samples were purified on Sep-Pak C_{18} cartridges (Waters) by washing with 15 mL of H_2O and eluting with 5 mL methanol. Samples containing ovalbumin as acceptor were precipitated with 1 mL of cold 15% trichloroacetic acid and 5% phosphotungstic acid solution and treated as described previously [19]. The amount of [^{14}C]Gal transferred to the acceptors was measured in a liquid scintillation β-counter (Rackbeta, Pharmacia).

Galactosidase assay

The disaccharide standards [^{14}C]Galβ1,3GlcNAc-β-O-p-nitrophenol (β-OpNP) and [^{14}C]Galβ1,4GlcNAc(β-OpNP) were synthesized using the β3GalT-I [11] and β4GalT [19] enzymes. Approximately 20 pmol of the purified standards and 20 pmol of [^{14}C]-labeled β3GalT-V reaction product were digested for 24 h at 37 °C with 2 U of recombinant *Xanthomonas manihostis* α-galactosidase (New England Biolabs Inc.) in 50 m M sodium citrate, pH 6.5, 5 m M $CaCl_2$ and with 2 U of recombinant *X. manihostis* β-galactosidase (New England Biolabs Inc.) in 50 m M sodium citrate, pH 4.5, respectively. The reaction products were purified on Sep-Pak C_{18} cartridges and the amount of [^{14}C]Gal remaining bound to the acceptors was measured in the β-counter.

Northern blotting

Total RNA from SK-N-MC, HeLa, HL-60, A-431, Caco2, Colo205 and PANC-1 cell lines was isolated by the procedure of Chomczynski and Sacchi [20]. Five micrograms of each tissue RNA were separated on 1% agarose-formaldehyde gels. Gels were rinsed in diethyl pyrocarbonate -treated water for 1 h and either stained in ethidium bromide, 0.1 M ammonium acetate or transferred to Hybond-N-Plus membranes (Amersham). β3GalT mRNA in human tissues was detected by Northern blot analysis using commercially available multiple tissues poly(A)$^+$ RNA blots (Clontech). A *Bam*HI-*Xho*I

Image Previews

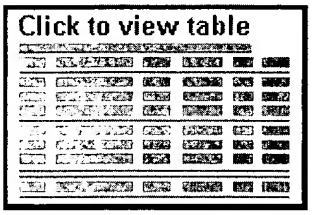

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Table 1. **Acceptor substrate specificity of the β3GalT-I and β3GalT-V enzymes.**

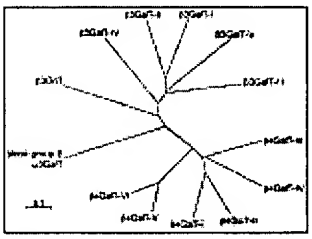

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Fig. 1. **Phylogenetic analysis of human galactosyltransferases.** Multiple alignment was performed on the...

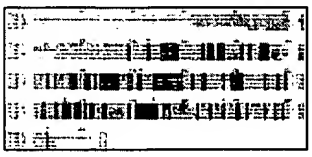

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Fig. 2. **Alignment of human β3GalT I, -II, -III and -V protein sequences.** The sequences of the four β3GalT...

956-bp fragment from the β3GalT-V gene was labeled with [α-^{32}P]CTP (Hartmann Analytics, Braunschweig, Germany) by random priming. Membranes were hybridized for 16 h at 68 °C according to the manufacturer, then washed in 0.1 × NaCl/Cit, 0.1% SDS up to 55 °C and exposed for three days on Biomax-MS film (Kodak) using the appropriate intensifying screens.

Results

Go to: Choose Go

The protein sequences of the β3GalT-I, -II and -III genes were compared to the EST division of GenBank using BLAST search algorithms [21]. The 338-bp human EST AJ003597 contained an open reading frame that was significantly similar to the three query sequences. The gene encoding the AJ003597 EST was identified within the PAC clone 70I24 (AF064860), which corresponds to a 165-kb stretch of chromosome 21 mapping to position 21q22.3. This gene included an open reading frame of 933 bp, which encoded a protein of 310 amino acids that was tentatively named β3GalT-V (GenBank accession number AF145784). The β3GalT-V protein contained a single membrane-spanning domain as deduced by Kyte-Doolittle hydropathy analysis [22]. The putative transmembrane domain began at amino acid position 8 and was 21 amino acids long, thus supporting a type-II transmembrane topology typical of most glycosyltransferases [3]. Phylogenetic analysis of the human galactosyltransferase enzymes indicated that β3GalT-V was most closely related to the β3GalT proteins, whereas these genes probably diverged consecutively to gene duplication events (Fig. 1).

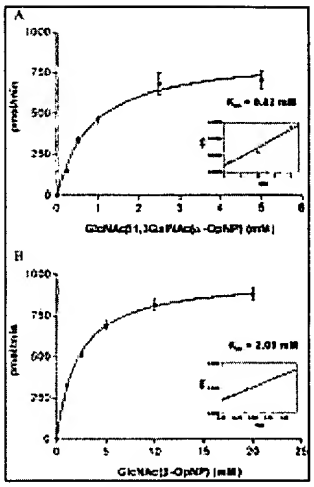

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Fig. 3. **Kinetic analysis of β3GalT V.** The K_m values for the acceptors GlcNAcβ1,3GalNAc(α-OpNP) (A) and Glc...

The β3GalT-V protein shared 34%, 27%, 31% and 23% sequence identity with β3GalT-I, -II, -III and -IV enzymes, respectively. The comparison with the β3GalT-I, -II, -III proteins, which were used as the query for the retrieval of β3GalT-V, indicated several conserved regions within the C-terminal two-thirds of the protein sequence, which corresponds to the catalytic domain according to the typical organization of glycosyltransferases (Fig. 2). As expected, the N-terminal third encoding the cytoplasmic, transmembrane and stem domains, did not show much similarity to the other β3GalT enzymes. The β3GalT-V enzyme included all sequence motifs conserved among β3GalT proteins [11-14]. In addition, six cysteine residues, which are conserved in β3GalT-I to -III enzymes but absent in the structurally related β3GalT-IV and β3GnT [23] enzymes, are also found in the β3GalT-V amino acid sequence, indicating that this enzyme is mostly related to the group of β3GalTs that transfer Gal to GlcNAc-based acceptors.

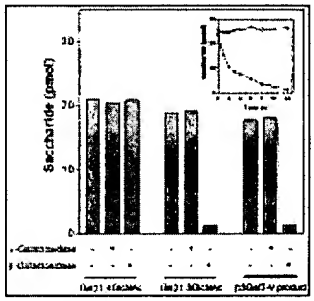

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Fig. 4. **Galactosidase digestion of the β3GalT-V reaction product.** The [^{14}C]Galβ1,3GlcNAc(β-OpNP) and [^{14}...C...

To confirm the galactosyltransferase activity of the cloned gene, we constructed a recombinant baculovirus including the human β3GalT-V gene by applying a transposon-mediated recombination system [24]. We have compared the galactosyltransferase activity in the lysate of Sf9 insect cells infected with recombinant baculoviruses expressing the β3GalT-V and the β3GalT-I enzyme [11]. Both β3GalT enzymes displayed a preference towards GlcNAc-based acceptors and failed to transfer Gal to GalNAc (Table 1). However, β3GalT-V was able to transfer Gal to the O-linked *core3* substrate whereas β3GalT-I did not use this disaccharide acceptor efficiently. Another difference between both enzymes was detected when using the N-linked glycoprotein ovalbumin as an acceptor substrate, as β3GalT-V catalyzed only a small incorporation of Gal onto this acceptor protein. Assays performed using UDP-GalNAc and UDP-GlcNAc as donors revealed that the two enzymes exhibit neither N-acetylglucosaminyltransferase nor N-acetylgalactosaminyltransferase activity (data not shown). As β3GalT-V was able to transfer Gal to GlcNAc(β-OpNP) and GlcNAcβ1,3GalNAc(α-OpNP), we have determined the K_m of the enzyme for these two acceptors. Saturation curves yielded apparent K_m values of 0.82 m M and 2.09 m M for GlcNAcβ1,3GalNAc(α-OpNP) and GlcNAc(β-OpNP), respectively, thereby indicating the preference of β3GalT-V for the O-linked *core3* acceptor substrate (Fig. 3).

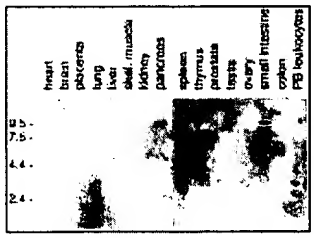

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Fig. 5. **Expression pattern of the β3GalT-V gene in adult human tissues.** Each lane represents about 2 µg o...

The type of glycosidic linkage catalyzed by the β3GalT-V enzyme was analyzed by *X. manihostis* galactosidase digestion [25]. The product of the reaction catalyzed by β3GalT-V using the acceptor GlcNAc(β-OpNP) and the donor UDP-Gal was purified on

Sep-Pak C_{18} cartridges and digested with galactosidases. Like the disaccharide Gal β1,3GlcNAc (β-OpNP), the β3GalT-V reaction product was cleaved by the *X. manihostis* β-galactosidase whereas it was found to be insensitive to α-galactosidase treatment (Fig. 4). In comparison, the disaccharide Galβ1,4GlcNAc(β-OpNP) remained intact after β-galactosidase digestion, thus showing the specificity of this galactosidase towards the β1,3 linkage (Fig. 4).

The expression of the β3GalT-V gene was investigated in adult human tissues by Northern blot analysis. β3GalT-V transcripts were detected in small intestine, pancreas and testis (Fig. 5). Two transcripts of 7 kb and of ≥ 10 kb were visible in small intestine. Only the mRNA larger than 10 kb was detected in pancreas and testis, although other mRNAs may be present below the detection limit considering the weak hybridization signal observed in those tissues. By comparison, a large transcript of 7 kb was also detected for the β3GalT-I gene [11, 14]. The expression pattern of the β3GalT-V gene was also analyzed in tumor cell lines of different tissue origin. β3GalT-V transcripts were visible in the pancreatic adenocarcinoma PANC-1 line and in the colorectal adenocarcinoma Colo205 cells (Fig. 6). By contrast, β3GalT-V mRNA was not detectable in another colorectal adenocarcinoma line, Caco2, and in neuroblastoma SK-N-MC, squamous carcinoma A-431, promyelocytic leukemia HL-60 and cervical carcinoma HeLa cells.

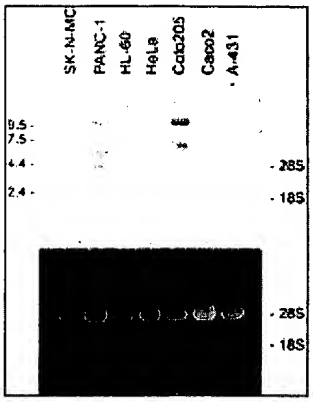

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Fig. 6. **Expression of the β3GalT-V gene in tumor cells.** Total RNA (5 µg) from tumor cell lines was separa...

Discussion Go to: Choose Go

The recent characterization of β3GalT genes has underlined the structural differences between these genes and the other members of the galactosyltransferase family. It appears that β3GalT enzymes share conserved sequences that are not found in β4GalT and α3GalT proteins. These unique structural features have been exploited to identify additional β3GalT genes, as illustrated in the present report describing the cloning of the human β3GalT-V. Besides the amino acid sequence conservation, the β3GalT-V gene also displayed a similar genomic organization, i.e. the entire protein-coding sequence is included in a single exon, as deduced by examination of the genomic PAC clone 70I24. In addition, as observed for β3GalT-I, the β3GalT-V transcripts were unexpectedly large for an open reading frame of 0.9 kb.

The same enzyme as the one reported here has been recently described by others under the name β3Gal-T5 [26]. That work showed the capability of the β3GalT-V to direct the expression of type 1 Lewis antigens when transfected in Namalwa lymphoma cells. The activity of the β3GalT-V enzyme towards GlcNAc was confirmed by our experiments based on the recombinant expression of β3GalT-V in insect cells. In addition, the expression of recombinant β3GalT-V revealed the preference of the enzyme for the O-linked *core3* GlcNAcβ1,3GalNAc acceptor. β3GalT-V was the only β3GalT enzyme identified to date capable of efficiently transferring Gal to this acceptor [11]. Also, the poor activity of β3GalT-V towards the N-linked glycoprotein ovalbumin supported the preference of this enzyme for mucin-type glycoprotein acceptors. These findings suggest that β3GalT-V probably represents the same enzyme as the β3GalT previously isolated from pig trachea [18]. β3GalT-V transcripts were not detected in lung tissue as analyzed by Northern blotting. However, this may indicate that the β3GalT-V gene expression is restricted to tracheal tissue, which was not represented in the source of lung RNA analyzed.

The β3GalT-V gene was expressed in small intestine, pancreas and testis but not in differentiated colon tissue, whereas β3GalT-V transcripts were detected in colonic adenocarcinoma Colo205 cells. A β3GalT activity has been previously described in Colo205 cells [27-29] and in normal colonic mucosa [30]. The work of Isshiki *et al.* [26] demonstrated that this β3GalT activity, encoded by the β3GalT-V enzyme, was directed towards lacto-series structures, thereby contributing to the formation of type 1 carbohydrate chains. However, the absence of β3GalT-V transcript in Caco2 cells and in other colorectal cancer cell lines [26] indicates that the neo-expression of this gene is not a general feature of colonic adenocarcinomas. Caco2 and Colo205 cell lines will be useful in investigating the relationship between the expression of β3GalT-V and the

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Zhou, Dapeng, Berger, Eric G. & Hennet, Thierry (1999) Molecular cloning of a human UDP-galactose:GlcNAc β1,3GalNAc β1,3 galactosyltransferase gene encoding an O-linked core3-elongation enzyme. *European Journal of Biochemistry* **263** (2), 571-576. doi: 10.1046/j.1432-1327.1999.00541.x

changes in levels of type 1 chain carbohydrate structures such as Le^a and Le^b antigens occurring in colonic cancer.

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Acknowledgements

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Abbreviations Go to: Choose Go

β3GalT β1,3 galactosyltransferase
β4GalT β1,4 galactosyltransferase
α3GalT α1,3 galactosyltransferase
Bn benzyl
pNP *p*-nitrophenol
EST expressed sequence tag.

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Footnotes

Note: the nucleotide sequence reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number AF145784.

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